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# Demonstration that *fbiC* Is Required by *Mycobacterium bovis* BCG for Coenzyme $F_{420}$ and FO Biosynthesis

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**Using the nitroimidazopyran-based antituberculosis drug PA-824 as a selective agent, transposon-generated *Mycobacterium bovis* strain BCG (*M. bovis*) mutants that could not make coenzyme $F_{420}$ were identified. Four independent mutants that could not make $F_{420}$ or the biosynthesis intermediate FO were examined more closely. These mutants contained transposons inserted in the *M. bovis* homologue of the *Mycobacterium tuberculosis* gene Rv1173, which we have named *fbiC*. Complementation of an *M. bovis* FbiC$^-$ mutant with *fbiC* restored the $F_{420}$ phenotype. These data demonstrate that *fbiC* is essential for $F_{420}$ production and that FbiC participates in a portion of the $F_{420}$ biosynthetic pathway between pyrimidinedione and FO. Homologues of *fbiC* were found in all 11 microorganisms that have been fully sequenced and that are known to make $F_{420}$. Four of these homologues (all from members of the aerobic actinomycetes) coded for proteins homologous over the entire length of the *M. bovis* FbiC, but in seven microorganisms two separate genes were found to code for proteins homologous with either the N-terminal or C-terminal portions of the *M. bovis* FbiC. Histidine-tagged FbiC overexpressed in *Escherichia coli* produced a fusion protein of the molecular mass predicted from the *M. bovis* BCG sequence (~95,000 Da), as well as three other histidine-tagged proteins of significantly smaller size, which are thought to be proteolysis products of the FbiC fusion protein.**

The structure of coenzyme $F_{420}$ (a 7,8-didemethyl-8-hydroxy 5-deazaflavin electron transfer agent that is present in few microorganisms) was first determined in studies with the methanogenic *Archaea* (13, 14). This coenzyme had been previously purified from a methanogen, and its spectral and fluorescence properties were described in detail by Cheeseman et al. (6), who gave it its name (for a factor that absorbed maximally in visible wavelengths at 420 nm). However, an earlier report by Cousins described a yellow compound of unknown structure from *Mycobacterium smegmatis* that, based on its UV-visible spectrum, was almost certainly $F_{420}$ (11). Naraoka et al. reported that $F_{420}$ was present in *Mycobacterium avium* (37), and it was subsequently discovered that $F_{420}$ was present in *Mycobacterium tuberculosis* (12) and all other mycobacteria examined (2, 43). *Methanobacterium thermoautotrophicum* $F_{420}$ contains two glutamyl residues (14), but *Mycobacterium* species contain primarily five- and six-glutamyl forms of $F_{420}$ ($F_{420}$-5 and $F_{420}$-6) (2). The structure of $F_{420}$-5 is shown in Fig. 1A.

In *Mycobacterium* and *Nocardia* species, $F_{420}$ is used by $F_{420}$-dependent glucose-6-phosphate dehydrogenase (42, 43) and is required for activation of the experimental antituberculosis drug PA-824 by *M. tuberculosis* and *Mycobacterium bovis* strain BCG (hereafter referred to as *M. bovis*) (48). It is likely that other $F_{420}$-dependent reactions will be discovered in mycobacteria, since genes corresponding to several proteins with homology to $F_{420}$-dependent enzymes from other organisms are present in the *M. tuberculosis* genome (41). In *Archaea*, $F_{420}$ is required for hydrogenase, formate, methylene-tetrahydromethanopterin, and alcohol dehydrogenases, methylene-tetrahydromethanopterin reductase, and quinone oxidoreductase (20, 22, 24, 28, 31, 50). $F_{420}$ is used by *Streptomyces* for lincomycin and tetracycline biosynthesis (8, 29, 34, 46), and possibly in mitomycin C biosynthesis (32). The *Archaea M. thermoautotrophicum* and *Halobacterium* sp., the green alga *Scenedesmus acutus*, and the cyanobacterium *Synechocystis* sp. use $F_{420}$ in photolyase (16, 17, 26, 38).

In *M. thermoautotrophicum*, the pathway by which $F_{420}$ is made has been studied with labeling (15, 23, 45) and enzymatic approaches (19), which have allowed development of a hypothetical pathway, an overview of which is shown in Fig. 1B. Due to our interest in $F_{420}$ biosynthesis and in the metabolism of pathogenic mycobacteria, we have begun a study to identify the genes involved in $F_{420}$ biosynthesis in *Mycobacterium*. The only description of genes that are required for $F_{420}$ biosynthesis has been a recent report that homologues of the *M. tuberculosis* genes Rv3261 and Rv3262 are required for $F_{420}$ biosynthesis in *M. bovis* (7). We named these genes *fbiA* and *fbiB*, respectively. Here we report that transposon Tn*5367* insertion into the *M. bovis* homologue of the Rv1173 gene from *M. tuberculosis* creates mutants that cannot produce $F_{420}$ or the biosynthetic intermediate FO. We name this gene *fbiC* (for $F_{420}$ biosynthesis) and conclude that it is required for a step in the pathway prior to FO, before the deazaflavin ring is formed.

## MATERIALS AND METHODS

**Bacteria, plasmids, and culture conditions.** The plasmids and bacteria used in this study are given in Table 1. *M. bovis* was grown in Middlebrook 7H9 medium supplemented with 0.2% (vol/vol) glycerol, 10% (vol/vol) Bacto Middlebrook ADC Enrichment, 0.05% Tween 80 (vol/vol; liquid medium only), and 1.5% agar (plate medium only). With *Escherichia coli* JM109 as the recombinant host, pGEM, pSMT3, and pHAT-12 were the vectors. *E. coli* was grown at 37°C on Luria-Bertani medium; ampicillin (100 μg/ml for work with pGEM and 200 μg/ml with pHAT), isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 80 μg/ml) were included for recombinant selection and identification.

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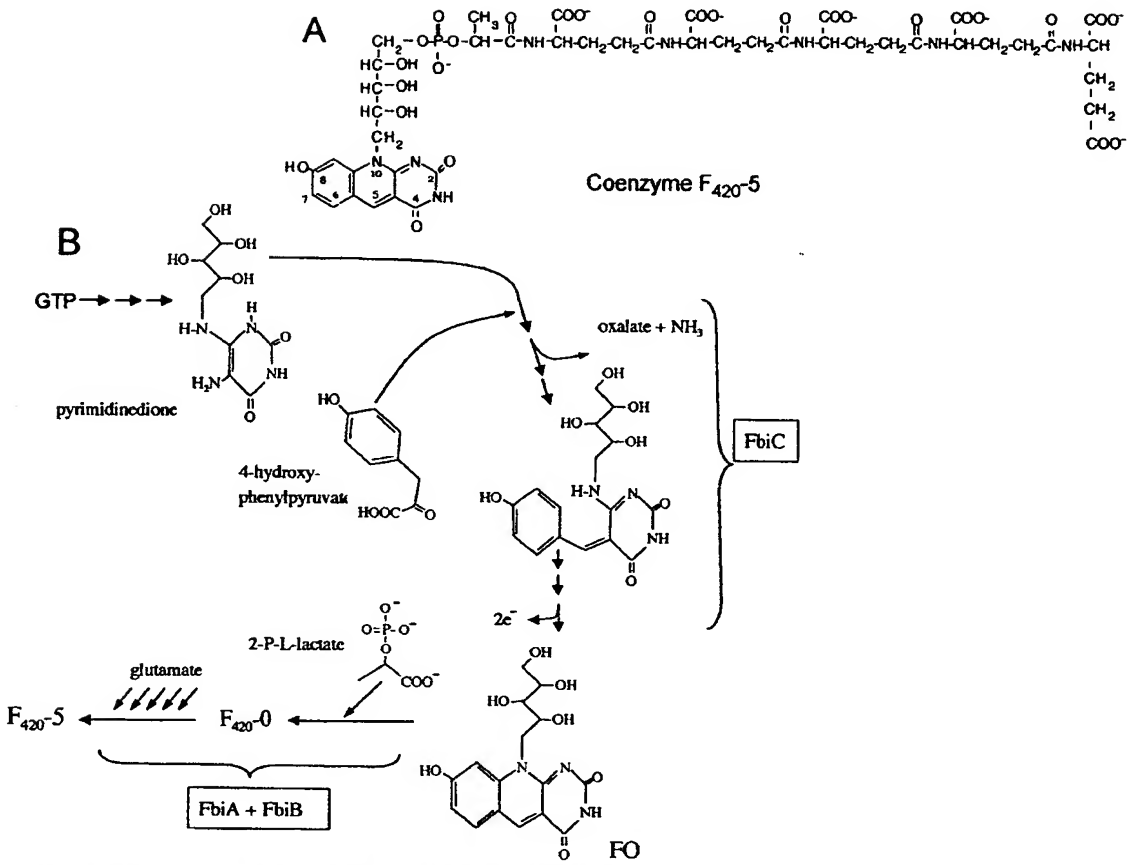


FIG. 1. Structure of $F_{420}$-5 and an overview of the hypothetical pathway for $F_{420}$ biosynthesis. (A) $F_{420}$-5 (2); (B) overview of the hypothetical pathway for $F_{420}$ biosynthesis (2, 7, 15, 19, 23, 45). FbiA and FbiB are clearly involved in the conversion of FO into $F_{420}$; evidence supports FbiA acting earlier in the path than FbiB, but the specific reactions catalyzed by these enzymes are not known.

**HPLC.** Cell extracts were prepared for high-performance liquid chromatography (HPLC) by resuspending wet cell pellets in approximately the same volume of 25 mM sodium acetate (pH 4.7) as the pellet volume, followed by heating at >90°C for 15 min and centrifugation in a microcentrifuge. $F_{420}$ and FO were separated by HPLC by methods similar to that of Gorris and van der Drift (18), with modifications, as recently described (7).

**General molecular biology techniques.** Chromosomal DNA was purified according to the method of Husson et al. (21). Wizard minicolumns from Promega (Madison, Wis.) were used for pGEM- and pSMT3-based plasmid purification, and QIAquick kits (Qiagen) were used for PCR cleanup and gel extraction with pHAT constructs. All PCR cloning amplification was done with *Pfu* polymerase, except for the addition of an A overhang with *Taq* polymerase in pGEM cloning. Sequencing was conducted at the University of Iowa DNA Facility using an Applied Biosystems 373A DNA sequencer.

**Transposon mutagenesis and gene identification.** Transposon insertion mutants in *M. bovis* were created with the plasmid pPR29 as described by Pelicic et al. (40), but with the incorporation of PA-824 selection (7). The nitroimidazopyran PA-824 (used to select for mutants unable to make $F_{420}$ [$F_{420}^-$ mutants]) was a gift of the PathoGenesis Corporation (48). Colonies that survived this selection were grown and analyzed by HPLC for the presence of 5-deazaflavins. Transposon insertion sites were found as recently described (7).

**Complementation of the *fbiC* mutant.** PCR to amplify *fbiC* and the surrounding region was performed, the products were analyzed by agarose gel electrophoresis, and the expected 2,976-bp band was cloned into pGEM, resulting in pFbiC. One round of sequencing confirmed that *fbiC* had been cloned. pFbiC was amplified in *E. coli* and used as the template for PCR amplification of a 2,750-bp segment using primers which contained *Pst*I and *Hin*dIII sites. This PCR product was cut by *Pst*I and *Hin*dIII, and the product was inserted into *Pst*I- and *Hin*dIII-digested pSMT3 to make p1173. p1173 was used as an expression vector to complement insertion mutants. Following electroporation of 1 or 2 μg of p1173 into the mutants, transformants were selected for using hygromycin-supplemented liquid or agar media (50 μg/ml for *M. bovis*). Electroduction and/or electroporation to confirm that pSMT3 containing an insert was present in complemented cells was conducted using a modification of the method described by O Gaora (39), as recently described (7).

**Expression of histidine-tagged FbiC.** To create a vector to overproduce FbiC, *fbiC* was amplified from p1173 by using primers which contained *Sac*I sites near their 5′ end, and this product was inserted into *Sac*I-digested pHAT-12 such that the histidine affinity tag (HAT) was in frame with FbiC. Plasmids derived from *E. coli* transformants were examined with one round of sequencing to confirm correct insertion, and one such *fbiC* clone (pHAT1173) was then completely sequenced.

Western blotting to identify proteins containing the HAT tag was conducted according to the manufacturer's instructions by using a polyclonal anti-HAT antibody (Clontech, Palo Alto, Calif.) that recognizes epitopes throughout the HAT tag. The secondary antibody was a polyclonal goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase, which was used with the Immun-Star Chemoluminescent substrate (Bio-Rad). Protein bands from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein fractions which contained HAT-tagged proteins were identified with Kodak BioMax MR photographic film after 10- to 30-s exposures. *E. coli* JM109 carrying an expression vector coding for HAT-tagged dihydrofolate reductase (DHFR; Clontech) was used as a positive control, and *E. coli* JM109 with no expression vector was used as a negative control.

To study HAT-tagged FbiC production by cells carrying pHAT1173, a 1-liter culture was grown in a rotary shaker at 37°C overnight to an optical density at 600

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description or DNA sequence | Source or reference |
|---|---|---|
| Strains | | |
| *M. bovis* BCG | Montreal *M. bovis* BCG strain | C. Kendall Stover, PathoGenesis |
| *E. coli* JM109 | RecA$^-$, recombinant vector host strain | Promega |
| *M. bovis* FbiC$^-$-1 | BCG strain with Tn*5367* inserted in *fbiC* | This study |
| *M. bovis* FbiC$^-$-2 | BCG strain with Tn*5367* inserted in *fbiC* | This study |
| *M. bovis* FbiC$^-$-3 | BCG strain with Tn*5367* inserted in *fbiC* | This study |
| *M. bovis* FbiC$^-$-4 | BCG strain with Tn*5367* inserted in *fbiC* | This study |
| Plasmids | | |
| pGEM | T/A PCR cloning vector | Promega |
| pSMT3 | Expression vector for complementation in mycobacteria; carries hygromycin resistance marker | Larry Schlesinger (39) |
| pPR29 | *ts-sacB* delivery plasmid pPR27 with a Tn*5367* insert (*ori*M cannot function at 39°C) | Brigitte Gicquel, Institut Pasteur (33, 40) |
| pFbiC | pGEM containing *fbiC* as 2,976-bp insert | This study |
| p1173 | pSMT3 containing *fbiC* as 2,750-bp insert | This study |
| pHAT-12 | Cloning vector for addition of HAT tag to the N terminus; Amp$^r$ | Clontech |
| pHAT DHFR | Positive control expression vector with murine dihydrofolate reductase gene tagged with HAT at the N terminus | Clontech |
| pHAT1173 | pHAT-12 with *fbiC* insert as *Sac*I fragment | This study |
| Transposon | | |
| Tn*5367* | IS*1096* with kanamycin resistance cassette | Brigitte Gicquel, Institut Pasteur (3, 33, 40) |

nm of −1, followed by addition of IPTG to 1 mM and further incubation for 2 h. The entire culture was then frozen at −65°C overnight, thawed in warm water, and centrifuged to separate cells from the medium. Cell pellets were resuspended in 4 ml of cold extraction buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0) per 50 ml of original culture volume, vortexed for 2 to 3 min, and then incubated with lysozyme (0.75 mg/ml) for 45 min at room temperature with gentle agitation. Crude lysate (2 ml) was transferred to 2-ml screw-cap conical tubes to which zirconia and silica beads (0.1-mm diameter; Biospec Products) had been added to a height of 1 to 2 mm. The cells were then disrupted in a Mini-Beadbeater (Biospec Products) by shaking at 5,000 rpm for 30 s; tubes were kept on ice except when they were in the Beadbeater. This lysate was centrifuged at 10,000 × *g* for 20 min at 4°C to separate soluble and insoluble material. The resulting supernatant (cleared lysate) was stored on ice, and the pellet was resuspended in 1.5 ml of cold extraction buffer by shaking for 3 s at 5,000 rpm in the Beadbeater; the beads were allowed to settle, and the resuspended material was removed and placed on ice. Talon resin (a Cobalt-complexed resin made by Clontech that specifically binds the HAT tag) was used to purify HAT-tagged FbiC. Samples were bound to the resin, which was then washed with buffer and eluted with 100 and 200 mM imidazole, according to the manufacturer's instructions. In some cases, a protease inhibitor cocktail suitable for use with polyhistidine-tagged protein (P8849; Sigma) was used in cell breakage and all subsequent Talon steps. Samples of the culture supernatant, the cleared lysate, the resuspended lysed cell pellet, and fractions from Talon purification steps were mixed with an equal volume of 2× SDS-PAGE sample buffer (90 mM Tris HCl [pH 6.8], 20% glycerol, 2% SDS, 0.02% bromphenol blue), heated at 95°C for 5 to 10 min, and then analyzed by SDS-PAGE followed by Coomassie blue staining or Western blot analysis.

**Computer analysis of sequences.** Comparison of derived amino acid sequences to the protein database sequences was performed by the National Center for Biotechnology Information (NCBI) BlastP program (1). FbiC homologue sequences were compared by using the ClustalW (49) multiple-alignment program with default settings, available at the Baylor College of Medicine Search Launcher (http://dot.imgen.bcm.tmc.edu). Relationships of protein sequences were examined by the NCBI COGs and CD programs. The Sanger Centre TBLASTN program was used to locate and analyze the *M. bovis*, *M. leprae*, and *Streptomyces coelicolor* sequences that were homologous with Rv1173 and the surrounding regions (http://www.sanger.ac.uk). Preliminary sequence data from the Department of Energy Joint Genome Institute (JGI; at http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html) were used to locate *fbiC* homologues in *Nostoc punctiforme*, *Synechococcus* sp., *Thermobifida fusca*, and *Methanosarcina barkeri* by conducting a TBLASTN analysis of *M. bovis fbiC* at the corresponding organism-specific JGI server.

**Nucleotide sequence accession number.** The nucleotide sequence data for *fbiC* from *M. bovis* BCG have been deposited in GenBank under accession number AF479769.

## RESULTS AND DISCUSSION

***M. bovis* mutants with an insertion in the Rv1173 homologue make neither FO nor $F_{420}$.** Following transformation with pPR29, *M. bovis* transformants were grown for 4 to 6 weeks at 32°C in medium containing gentamicin and kanamycin to select for plasmid-containing cells. Cells from the liquid medium were then spread onto agar medium containing kanamycin, sucrose, and PA-824, and the plates were incubated at 39°C. Kanamycin selected for those bacteria containing the kanamycin marker in the transposon. Sucrose selected for transposons that had inserted into the chromosome and for cells that did not contain either the plasmid-borne *sacB* gene or this gene inserted into the chromosome; the temperature of 39°C also restricted plasmid replication, due to its temperature-sensitive origin of replication. PA-824 selected for cells that had lost the ability to activate PA-824 (7, 48). As shown previously (7), a significant subset of PA-824-resistant mutants was expected to be defective in $F_{420}$ biosynthesis. Four such $F_{420}$- mutants had independent insertions in the *M. bovis* homologue of the *M. tuberculosis* gene Rv1173. The insertion locations were throughout the gene, as shown in Fig. 2, from very near the N terminus to the C-terminal portion. None of the four mutants made FO or $F_{420}$. Figure 3 illustrates the HPLC profile of the wild-type *M. bovis* and one of the $F_{420}^-$ and FO$^-$ mutants. We have named this Rv1173 homologue gene *fbiC* (for $F_{420}$ biosynthesis).

**Complementation of $F_{420}^-$ mutants with *M. bovis fbiC*.** A 2,976-bp fragment containing *fbiC* from *M. bovis* was PCR cloned, and a 2,750-bp segment which contained the entire Rv1173 homologue gene was subcloned into pSMT3, creating

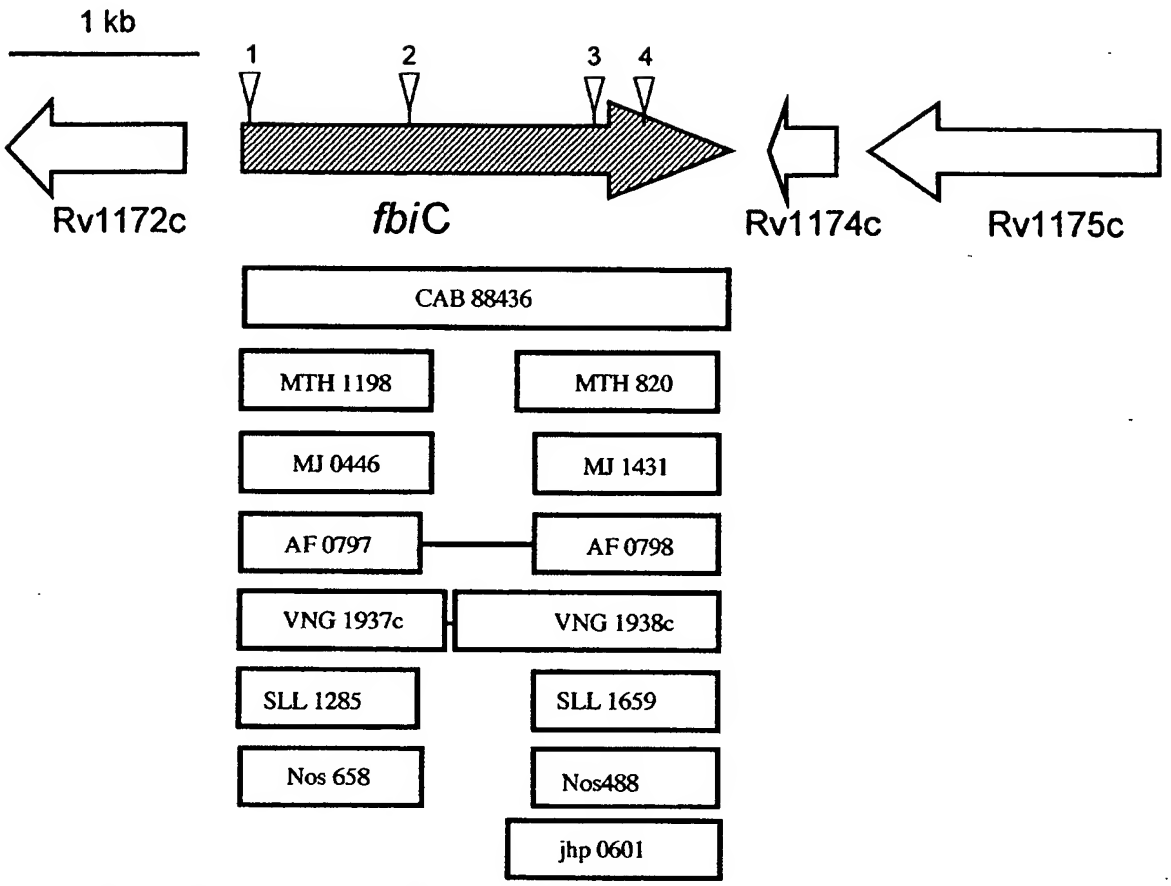


FIG. 2. Gene arrangement of the *fbiC* region in *M. bovis*, transposon insertion sites, and alignment of this gene with selected homologues from other microorganisms. Triangles indicate transposon insertion sites. Abbreviations: CAB, *S. coelicolor*; MTH, *M. thermoautotrophicum*; MJ, *M. jannaschii*; AF, *A. fulgidus*; Vng, *Halobacterium* sp.; SLL, *Synechocystis* sp.; Nos, *N. punctiforme*; jhp, *H. pylori*. Numbers refer to gene designations in the corresponding genome sequences, or contig number in the case of *Nostoc*. The figure is approximately to scale, with boxes indicating the sizes of homologous genes.

p1173. This plasmid was transformed into mutant FbiC$^-$-3. As shown in the bottom panel of Fig. 3, the ability to make $F_{420}$ and FO was fully restored by this complementation, confirming that the mutant phenotype resulted specifically from the insertion in *fbiC*.

**Production of histidine-tagged FbiC in *E. coli*.** The *M. bovis* *fbiC* gene was successfully cloned in frame into pHAT, creating the histidine-tagged expression construct pHAT1173. The *fbiC* gene in pHAT1173 was found by complete sequencing to be 100% identical to the known *M. bovis* BCG sequence in the Sanger Centre database, proving that no errors were introduced by *Pfu* DNA polymerase cloning. As shown in Fig. 4a, when cleared lysate from *E. coli* carrying pHAT1173 was examined by SDS-PAGE, there appeared to be no major Coomassie blue-stained band that was the expected 97,186-Da size of the HAT-tagged FbiC (92,486-Da FbiC plus 4,700-Da tag and linker). However, a 95-kDa band appeared on the corresponding Western blot of the cleared lysate sample (Fig. 4b, lane 3); the same band was seen in the culture medium taken from some cultures (Fig. 4b, lane 2). The lysate pellet had large amounts of HAT antibody-reactive material, suggesting the presence of insoluble FbiC inclusion bodies. The 95-kDa protein from cleared lysate bound in its native state to Talon resin and eluted with 100 mM imidazole (Fig. 4c and d). We conclude that this protein is the His-tagged FbiC. However, several other bands were present with smaller molecular weights (55, 45, and 10 kDa) which were also very reactive in the HAT Western blot (Fig. 4b, c, and d). Evidence is strong that these bands represent truncated versions of HAT-tagged FbiC, since two independent methods (HAT antibody recognition and Talon resin binding) indicate that the HAT motif is present. Also, the DHFR-positive control (cleared lysate of *E. coli* JM109 carrying the DHFR gene cloned into the same pHAT vector) shows the absence of such reactive bands, providing evidence that they arise from HAT-tagged *fbiC*. Examination of Western blots produced from cleared lysate and Talon purification fractions processed with and without protease inhibitors suggested that protein degradation was responsible for the appearance of the smaller Western blot bands. Unprocessed cleared lysate sampled soon after its preparation showed less intense and smaller bands (Fig. 4b, lane 3) than those of Talon-processed samples (Fig. 4c and d). The 10- and 55-kDa

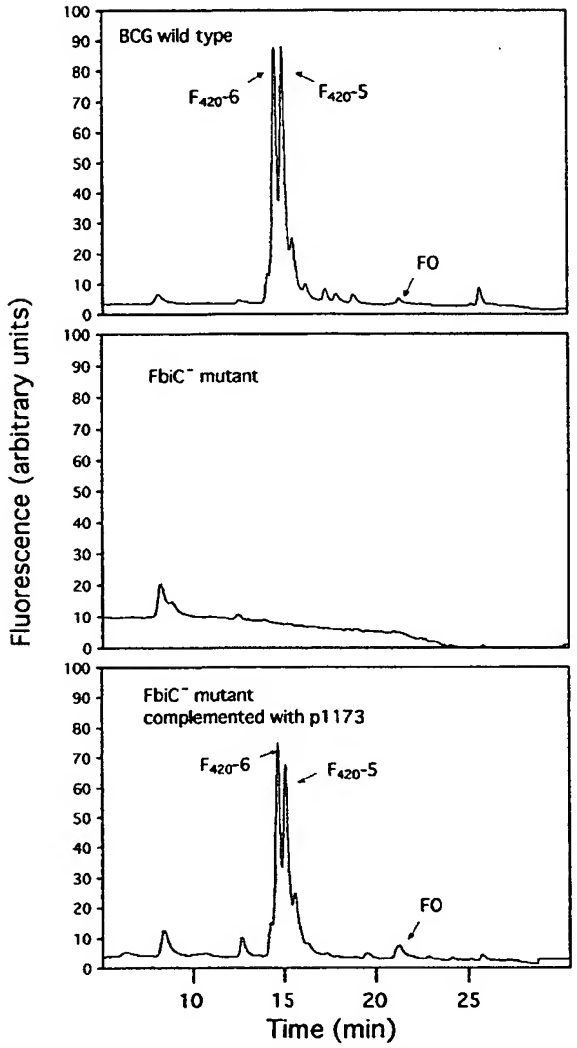


FIG. 3. HPLC profiles of *M. bovis* wild type, an $F_{420}^-$ mutant (FbiC⁻), and an FbiC⁻ mutant complemented with *fbiC*.

bands were the most intense if the imidazole-eluted Talon fractions were processed without protease inhibitors (Fig. 4c, lanes 5 to 9), but when processed with inhibitors, the 95-kDa band was the most intense and very little of the 10-kDa band was visible (Fig. 4d, lanes 6 to 8). This degradation is problematic since yield of the full-size protein is lowered, and the suitability of even the full-sized protein for chemical analysis and enzyme assay is uncertain. The degradation may arise from an aggressive attack on properly folded *M. bovis* FbiC by *E. coli* proteases, or it may result from a greater sensitivity to proteases by FbiC that has not been properly folded. Despite the degradation problems, production of a protein of the expected size establishes that the full length of *fbiC* from *M. bovis* can code for one protein. This is important since homologues from some organisms code for two separate proteins corresponding to the N-terminal and C-terminal portions of *fbiC*, as described below.

**Comparison of *M. bovis* FbiC with homologues from other $F_{420}$-producing species.** The arrangement of genes around *fbiC* from *M. bovis*, *M. leprae*, and *M. tuberculosis* H37Rv is identical to that shown in Fig. 2. BlastP analysis showed that, compared to *M. bovis*, the translated *fbiC* sequences from *M. tuberculosis* and *M. leprae* were, respectively, 100 and 87% identical at the amino acid level for the full length of the sequence when the low-complexity filter was not used. BlastP analysis of the *M. bovis* FbiC against protein databases (using a filter to avoid comparison of regions of low complexity, thus providing a conservative measure of protein similarity) revealed that the actinomycetes *S. coelicolor* (Sanger database) and *Thermobifida fusca* (JGI database) contained genes coding for proteins with high homology for the full length of the sequence (Table 2 and Fig. 2). BlastP analysis also showed good hits with a variety of shorter sequences. The best of the shorter-sequence hits were almost entirely with *Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*, *Methanosarcina barkeri*, *Archaeoglobus fulgidus*, and *Halobacterium* sp. The cyanobacteria *Nostoc punctiforme* and a *Synechocystis* species had good hits as well. The percentages of identity for these segments are shown in Table 2, and examples of the regions of greatest similarity are indicated in Fig. 2. The full genome sequences for all these organisms have been completed and annotated (5, 9, 10, 25, 27, 38, 44, 47) or at least fully sequenced (by the JGI for *Methanosarcina barkeri*, *Nostoc* sp., and *T. fusca*). The methanogens, mycobacteria, *S. coelicolor*, *A. fulgidis*, *Halobacterium* sp., and *Synechocystis* sp. have been reported to make $F_{420}$ (12–14, 17, 30, 36, 43), and it has been determined that *T. fusca* makes $F_{420}$ (L. Daniels, unpublished data). This agreement in homology is consistent with a role for FbiC in $F_{420}$ biosynthesis.

The sequence comparisons in Fig. 2 and Table 2 are complex, since there is similarity between the N-terminal and C-terminal portions of FbiC. The N-terminal portion of *M. bovis* FbiC has its best hits with proteins found only in $F_{420}$ producers but has weak hits with sequences corresponding to the C-terminal portion of FbiC from many $F_{420}$ producers. In contrast, the C-terminal portion of the *M. bovis* protein has its best hits with $F_{420}$ producer proteins (corresponding to the weak hits seen with the N-terminal segment of FbiC) but still has fairly good homology with a family of proteins in bacteria which cannot make $F_{420}$ (e.g., homology with *Helicobacter pylori* jhp0601, the most similar of the proteins from microbes that do not make $F_{420}$).

*Mycobacterium* species, *T. fusca*, and *S. coelicolor* have one gene for the FbiC N- and C-terminal regions (which should produce one protein, as demonstrated above with the *M. bovis* HAT-tagged FbiC). However, *A. fulgidus*, *M. barkeri*, and *Halobacterium* sp. have adjacent genes that code for two separate proteins over approximately the same sequence. *Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*, and the cyanobacteria have two nonadjacent genes that correspond to this region. We propose that FbiC should refer to the intact, full-length sequence and that the two domains of this large protein be called FbiC (N terminal) and FbiC (C terminal).

Since the transposon insertion sites in *fbiC* that cause a 5-deazaflavin⁻ phenotype are found in both N-terminal and C-terminal regions, it is possible that both domains are impor-

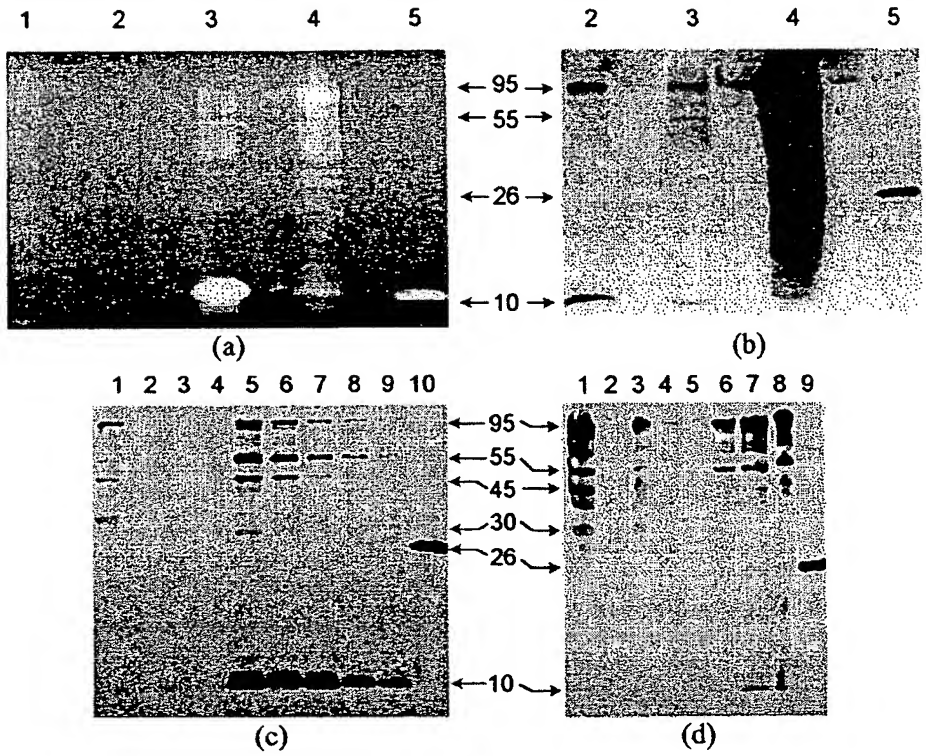


FIG. 4. Coomassie blue staining and Western blot analyses of FbiC expression and purification fractions. Samples taken from the lysates of an induced culture expressing FbiC were separated in duplicate by SDS–12% PAGE and either stained with Coomassie blue (a) or subjected to Western blotting using a HAT-specific antibody (b). Lane assignments for panels a and b: 1, Benchmark prestained protein ladder; 2, culture supernatant; 3, cleared lysate; 4, lysate pellet; 5, HAT-DHFR cleared lysate (control). (c) Samples taken from TALON purification of FbiC from the cleared lysate of an induced culture in the absence of protease inhibitors were separated and analyzed by Western blotting. Lane assignments for panel c: 1, lysate after TALON adsorption; 2 to 4, sequential wash fractions; 5 to 9, sequential elution fractions; 10, HAT-DHFR cleared lysate (control). (d) Samples from a purification of FbiC in the presence of protease inhibitors were separated and analyzed by Western blotting. Lane assignments for panel d: 1, cleared lysate; 2, blank; 3, lysate after TALON adsorption; 4 and 5, sequential wash fractions; 6 to 8, sequential elution fractions; 9, HAT-DHFR cleared lysate (control). Arrows marked with numbers represent estimated relative molecular masses in kilodaltons (kDa). Samples were loaded at 15 μl per well.

tant for $F_{420}$ biosynthesis. Alternatively, the insertions in the C-terminal region may interfere with the activity coded for by the N-terminal portion. This must be experimentally examined by making individual mutations in the two domains coded for by different genes in one of the $F_{420}$ producers. It is possible that the function of the C-terminal domain in $F_{420}$ producers is not unique for the production of $F_{420}$. Due to the essentiality of $F_{420}$ for the methanogens and *Archaeoglobus* (it is central to their energy generation pathways) and the economic barriers to using $F_{420}$ as a supplement in media, it is worth determining if *Halobacterium*, *Nostoc*, or *Synechocystis* species can be used to investigate the roles of these two separate genes in $F_{420}$ biosynthesis.

As shown in Fig. 5, *fbiC* in *T. fusca* is located in a cluster with *fbiA* and *fbiB*. As in *M. bovis*, *M. tuberculosis*, and *M. leprae*, a *whiB* homologue is located upstream of *fbiA*, except that in *T. fusca* another gene may be between the two. *T. fusca* is the only organism we have found where these three functionally related genes (*fbiABC*) are clustered. It is possible that such an arrangement is seen among some other actinomycetes, but in *S. coelicolor* (the only other nonmycobacterium actinomycete that has been fully sequenced), and in the three *Mycobacterium* species for which we have genome sequences, these three genes are not adjacent.

We have compared the N-terminal half and the C-terminal half of the *M. bovis* FbiC with homologous sequences from 10 of the known $F_{420}$ producers for which this sequence is available, using multiple alignment (data not shown). Several regions of very high identity are seen, especially an AGxlxiPFT TGlLvGIGE segment in the N-terminal comparison, and the tiPGTAAEiLxDxvR segment in the C-terminal comparison (amino acids always found are shown in uppercase letters, amino acids found most of the time are in lowercase letters, and x indicates that no one amino acid predominates). A group of three cysteines is seen in both N-terminal (txxCxxxCxYCxf) and C-terminal (NiNfTxiCxxxCxFCxF) regions.

**Consideration of the role of FbiC in $F_{420}$ biosynthesis.** The reaction catalyzed by FbiC must play a role in the early portion of the pathway shown in Fig. 1B, between the pyrimidinedione and FO, since no FO is made by the FbiC$^-$ mutants. The N-terminal portion of FbiC shows no great similarity to any protein of known function, but the most-similar known en-

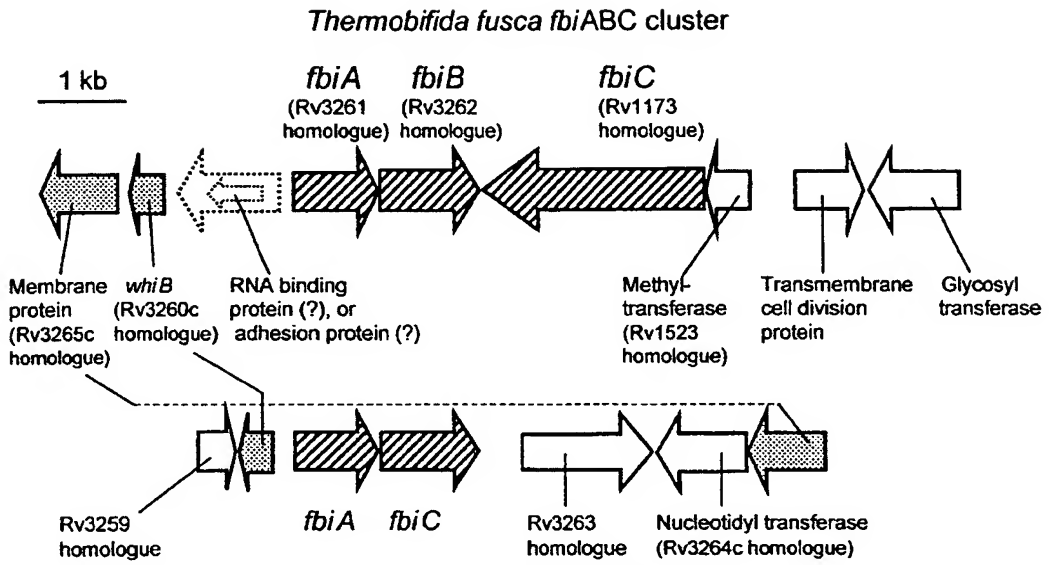


FIG. 5. Comparison of gene arrangement of the *fbiABC* cluster in *Thermobifida fusca* with the *fbiAB* region in *M. bovis*. Rv numbers refer to genes identified in *M. tuberculosis* H37Rv.

TABLE 2. BlastP comparison of homologues with *M. bovis* BCG FbiC[a]

| Homologue | Organism | % Identity with BCG FbiC: | |
|---|---|---|---|
| | | N-terminal domain | C-terminal domain |
| ML1492[b] | *M. leprae* | 84 | –[c] |
| ML1492[d] | *M. leprae* | – | 90 |
| SCD6.07[b] | *S. coelicolor* | 59 | 25 |
| SCD6.07[d] | *S. coelicolor* | 20 | 63 |
| TfFbiC[b,e] | *T. fusca* | 64 | 29 |
| TfFbiC[d,e] | *T. fusca* | – | 53 |
| MJ0446 | *Methanococcus* | 41 | – |
| MJ1431 | *Methanococcus* | 21 | 50 |
| MTH1198 | *M. thermoautotrophicum* | 39 | 21 |
| MTH820 | *M. thermoautotrophicum* | 27 | 45 |
| MsbFbiCa[e] | *M. barkeri* | 40 | – |
| MsbFbiCb[e] | *M. barkeri* | – | 47 |
| AF0797 | *A. fulgidus* | 40 | – |
| AF0798 | *A. fulgidus* | 23 | 43 |
| VNG1937c | *Halobacterium* sp. | 37 | – |
| VNG1938c | *Halobacterium* sp. | – | 32 |
| SLL1285 | *Synechocystis* sp. | 35 | – |
| SLL1659 | *Synechocystis* sp. | 23 | 41 |
| NosFbiCa[e] | *Nostoc* sp. | 34 | – |
| NosFbiCb[e] | *Nostoc* sp. | 24 | 42 |
| jhp0601 | *H. pylori* | – | 40 |

[a] Default BlastP analysis of homologue sequence versus *Mycobacterium tuberculosis* NCBI database, using a low-complexity filter. The low-complexity filter results in a conservative identity score, since sequences of low complexity are counted as not identical. Without the filtering, identity is usually higher; e.g., the *M. tuberculosis* and BCG sequences are 100% identical but are given as 89 and 81% in the BlastP results. Original papers describing genome or partial sequences are as follows: *M. tuberculosis* (9), *M. leprae* (10), *S. coelicolor* (44), *A. fulgidus* (27), *Halobacterium* sp. (38), *Methanococcus jannaschii* (5), *Methanobacterium thermoautotrophicum* (47), and *Synechocystis* sp. (25).

[b] The N-terminal half of the protein was used.

[c] –, no homologue found.

[d] The C-terminal half of the protein was used.

[e] Genes found at JGI site, not NCBI.

zymes identified by BlastP or COGS are BioB and ThiH, which participate in biotin and thiamine biosynthesis, respectively. However, these are not close matches, and *M. tuberculosis* has a putative BioB (Rv1589) with much greater homology to known BioB enzymes. No ThiH homologue could be identified in *Mycobacterium*, but it is likely that a putative ThiO (Rv0415) fulfills this function in *M. tuberculosis* (4, 35). Database comparison of the C-terminal portion of FbiC showed the best hits with hypothetical proteins, especially with probable iron-sulfur proteins (expect value [E], $\sim 10^{-40}$), and with very slight homology with two known proteins, ThiH and uroporphyrinogen decarboxylase (UroD or HemE). *M. tuberculosis* has a HemE homologue (Rv2678c) with high similarity to known HemE enzymes. We conclude that the inability of FbiC$^-$ mutants to make 5-deazaflavins does not result from interruption of biotin, thiamine, or heme biosynthetic genes and that the specific reaction catalyzed by *Mycobacterium* species FbiC is not clear based on sequence homologies. The development of assays for the reactions thought to be present in the early portion of the $F_{420}$ biosynthesis pathway shown in Fig. 1 will be very informative.

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